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STEINLEIN *et al.*

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Submission of English Translation of the Certified Copy of 35 U.S.C. § 119(a)-(d) Priority Document In Utility Application

Commissioner for Patents
Washington, D.C. 20231

Sir:

Submitted herewith is an English translation of the certified copy of Applicants' U.S.C. § 119(a)-(d) priority document, to overcome the date of a reference relied upon by the Examiner. Also submitted herewith is a Declaration by Jane Roberta Mann indicating that the translation of the certified copy is accurate.

Country	Priority Document Appl. No.	Filing Date
Germany	197 52 922.4	November 28, 1997

Prompt acknowledgment of this submission is respectfully requested.

Respectfully submitted,

STERNE, KESSLER, GOLDSTEIN & FOX P.L.L.C.

Michael A. Conlon
Reg No. 33851
for Eric K. Steffe
Attorney for Applicants
Registration No. 36,688

Date: 11/21/01

1100 New York Avenue, N.W.
Suite 600
Washington, D.C. 20005-3934
(202) 371-2600

P:\USERS\PChoi\0652\208\eng_trans_cert.wpd

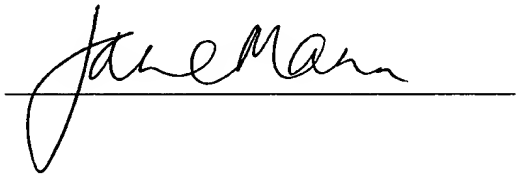
SKGF 8/23/01 mac

DECLARATION

I, Jane Roberta Mann, B.A., a Translator, of Frank B. Dehn & Co., 179 Queen Victoria Street, London, EC4V 4EL, do solemnly and sincerely declare that I have a competent knowledge of the English and German languages and that the following is a true and accurate translation of German Patent Application 197 52 922.4 filed on 28th November 1997 by Boehringer Ingelheim International GmbH.

I further declare that all statements made of my own knowledge are true and that all statements made on information and belief are believed to be true.

I acknowledge that wilful false statements and the like are punishable by fine or imprisonment, or both [18 U.S.C. 1001] and may jeopardize the validity of the application or any patent issuing thereon.



Signed this 3rd day of September, 2001

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BOEHRINGER INGELHEIM INTERNATIONAL GMBH
55216 Ingelheim am Rhein (FRG)

Process for measuring apoptosis

The invention relates to the field of biological test methods.

Apoptosis or programmed cell death (PCD) is a genetically controlled cellular suicide mechanism for selectively eliminating unwanted cells (1-4). PCD is an absolutely essential process in a number of biological processes, including embryonic and neural development, the regulation of the immune system, organogenesis, tissue homeostasis and the prevention of diseases such as tumour growth and virus infections. Apoptosis is characterised by blistering of the plasma membrane, shrinkage of the cells, condensation of the nucleus, endonucleolytic cleaving of genomic DNA into fragments of internucleosomal length and the formation of apoptotic bodies.

The methods currently available for investigating apoptosis are based on evaluating morphological changes at the cell level using light, electron or time-lapse microscopy in conjunction with fluorescent vital dyes, the use of annexin V, which can be used to monitor the loss of membrane phospholipid asymmetry during apoptosis (7), or they comprise assays for detecting DNA fragmentation by gel electrophoresis (8) or by *in situ* labelling of DNA strand breaks ("nick-end labelling") (TUNEL) (9).

However, most of these methods are either unsuitable for investigating the effect of genes which play a part in apoptosis, by transient transfection analysis, or, in the case of the TUNEL method, too expensive and laborious.

The problem of the present invention was to provide a new method of measuring apoptosis which overcomes the disadvantages of the known methods.

This problem was solved by a process for measuring apoptosis which is characterised in that

A) a population of mammalian cells is transiently transfected

ai) with a plasmid containing a DNA sequence of interest which is to be investigated as to whether it or the polypeptide expressed thereby has a pro- or anti-apoptotic activity,

aii) or with a plasmid containing a DNA sequence of interest which is to be investigated as to whether, or by means of which substances, its pro- or anti-apoptotic activity or the activity of the polypeptide expressed thereby can be modulated,

b) and with a plasmid containing a DNA coding for a fluorescent marker protein,

B) in that the cells are incubated in a suitable nutrient medium, optionally in the presence of a test substance, until the DNA sequence of interest or the expressed polypeptide has exerted its potential effect on the apoptosis,

C) in that the cells are harvested and fixed so that the fluorescent protein remains in the cells, while the DNA fragments formed during apoptosis are able to diffuse out of the cells,

D) in that the proportion of apoptotic cells is determined by measuring the DNA content,

E) in that the proportion of transfected cells is determined by measuring the cells having fluorescent marker protein,

F) and that by comparing the values obtained in steps D and E the proportion of apoptotic cells in the transfected subpopulation of the cells is determined.

The expression "DNA sequence of interest" (hereinafter also referred to as "apoptosis test gene") covers all DNA sequences which affect apoptosis directly or indirectly, as such or in the form of their translated products. Examples of genes which stimulate apoptosis are p53, bax, E1A, examples of apoptosis-inhibiting genes are bcl-2, bcl-x, E1B 19K, the latter group also including the so-called survival factors such as insulin-like growth factors (IGFs). Apoptosis genes of this kind and their activity have been described in summarising articles (e.g. 4, 23, 24).

The apoptosis test genes may be known or unknown genes or fragments thereof. By influencing apoptosis is meant both inducing and reinforcing as well as blocking and attenuating apoptosis.

The method according to the invention allows great variation, e.g. in terms of the markers used for determining the transfected cells and for the apoptosis, in terms of the plasmids and method of transfection used for transfecting the cells.

The method according to the invention has as one of its essential elements a fluorescent marker protein which serves to indicate the transient transfection of the cells.

The preferred marker protein is Green Fluorescent Protein (GFP). GFP mutants, which are tailored for FACS analysis and are suitable for use within the scope of the present invention, are known from the literature. One example of a suitable GFP mutant was described in (10) ("enhanced Green Fluorescent Protein", eGFP); however, within the scope of the present invention, other mutants may also be used which satisfy the condition that they do not influence cell metabolism, they remain located at the intracellular level and they deliver a measurable fluorescence signal, and in particular they are measurable using current methods of fluorescent activated throughflow cytometry (Fluorescent Activated Cell Sorting (FACS)).

Apart from Green Fluorescent Protein (GFP) other fluorescent marker proteins may also be used. Examples include Blue Fluorescent Protein (BFP) (26) and Yellow Fluorescent Protein (YFP) (25). The properties mentioned above for GFP mutants are essential for the suitability of a marker protein for use in the method according to the invention.

Potential marker proteins and the type and quantity of the plasmids coding for them which are to be used in the assay as well as the most suitable transfection method can be tested as follows, for example: the plasmids coding for the marker proteins are transiently transfected into mammalian cells, appropriately in the same cells and under the same conditions as are to be used for the method according to the invention. The

suitability of the transfected marker proteins is determined by series of measurements in which the transfection efficiency and the efficiency of the reproducible measurement are determined by FACS analysis.

The marker used for the apoptosis is a DNA-binding stain, e.g. propidium iodide (PI), which causes a reduction in fluorescence in the apoptotic subpopulation (14-17). This method of detection is based on the principle that the genomic DNA in cells is broken down endonucleolytically during apoptosis. The small DNA fragments diffuse out of the cell; the reduction in the DNA content to less than twice the set of chromosomes ("sub-2N") is an indication of apoptotic cells.

The reduced fluorescence of PI in cells which are undergoing apoptosis results in the appearance of a characteristic fluorescence peak ("sub-2N-peak") in the area of the G_0/G_1 region of the cell cycle.

Instead of propidium iodide, other DNA-binding stains may be used. Examples of suitable stains of this type are commercially available, e.g. DAPI (4',6'-diamidino-2-phenylindole), acridine orange, ethidium bromide. The most suitable stain can be determined by stimulating cells to apoptosis and then determining by FACS or microscopic analysis whether apoptosis can be reproducibly measured with the candidate stain.

One of the advantages of the method according to the invention is that the fluorescence of the marker protein and the DNA content can be measured simultaneously, preferably by FACS analysis. Suitable equipment is commercially available.

The invention is applicable to all mammalian cells which can be cultivated. It is a routine procedure for anyone skilled in the art to adjust the standard commercial FACS apparatus to different cell types.

For the transfection of the cells with marker gene on the one hand and the gene of interest on the other hand, all vectors which bring about efficient and reproducible expression in mammalian cells are suitable. Some of the numerous vectors available, including those which are commercially obtainable, contain regulatory sequences capable of achieving high expression rates in a number of mammalian cells. Examples include vectors which contain the CMV-(Cytomegalovirus), the SV40-(Simian virus), MSV (Moloney Sarcoma Virus)-promoter or other powerful promoters non-specific to cell type.

Identical or different vectors may be used as carriers for the marker gene and gene of interest; depending on the type of cell it may be advantageous to use vectors with different promoters, in order to avoid competition between the promoters during the transcription.

With regard to the transfection methods the invention is not subject to any restrictions; theoretically, all the methods known for the transient transfection of mammalian cells can be used, e.g. calcium phosphate, commercially obtainable cationic lipids such as lipofectamine or transfectam, methods based on receptor-mediated, Adenovirus-aided endocytosis, as described e.g. in WO 93/07283, for example using polyethyleneimine and psoralene/UV inactivated Adenovirus, as described in (21). The transfection method can be optimised, using series of tests in which

the transfection conditions, type of cell, nutrient medium etc. are varied, by transfecting with a fluorescent marker protein and determining the expression of the protein by FACS analysis. The optimised conditions for the marker protein are used for the co-transfection with the gene of interest.

After the transfection the cells are incubated in a suitable nutrient medium which is adapted to the particular type of cell. The cells may optionally be stimulated to apoptosis, particularly if the apoptosis test gene is to be investigated for any inhibition of apoptosis. Suitable apoptosis stimuli are known from the literature and commercially available; examples include staurosporin, daunomycin and etoposide. The incubation conditions and the suitability of an apoptosis stimulant are determined in preliminary trials. It is essential for the incubation, particularly its duration, that apoptosis has taken place to an extent which enables any change to be measured by measuring equipment, e.g. by FACS analysis.

The fixing step which is carried out after the incubation is essential to carrying out the process according to the invention.

The essential requirement for the fixing is that the conditions are such that the small subgenomic DNA fragments (internucleosomal fragments, i.e. those with a size of about 200 bp or a multiple thereof) occurring on apoptosis are able to diffuse out of the apoptotic cells, but at the same time the fluorescent marker protein remains in the cell. With the methods available up till now it was not possible to combine these measurements as the demands made on the fixing with respect to measuring the fluorescent marker protein on

the one hand and measuring the DNA content of the cells on the other hand were diametrically opposed and therefore seemed to be irreconcilable. The present invention makes it possible for the first time to carry out both measurements in the same cell population using a suitable fixing step.

In order to determine suitable fixing conditions the following procedure is preferably followed: first of all, the optimum fixing conditions for measuring the fluorescence of the marker protein on the one hand (strong fixing) and the optimum fixing conditions for measuring the DNA content of the cells on the other hand (weakest possible fixing) are determined independently of each other. Starting from the conditions with which the maximum measurements are obtained, the fixing conditions are modified in terms of the reagents (fixing reagent, salts, buffer), the concentration thereof and the fixing time in such a way that the efficiency is affected as little as possible when the two measuring operations are carried out simultaneously.

Preferably, the primary fixing is carried out with paraformaldehyde and the subsequent treatment (secondary fixing/permeabilisation) with ethanol; this treatment has proved most suitable in the tests performed. The primary fixing using 1 to 4 % (w/v), particularly 2 % paraformaldehyde takes place in an isotonic buffered saline solution. Standard solutions are suitable, such as 100 mM NaCl, 3 mM $MgCl_2$, 300 mM saccharose as well as standard commercial physiologically acceptable buffers.

Instead of paraformaldehyde it is also possible to use other reagents such as those which are conventionally

used, e.g. in immunohistochemistry. Examples of common fixing agents which can be found in the relevant textbooks (27) include formaldehyde or chloroform/acetone.

Instead of ethanol, which has proved particularly suitable under the conditions selected in the tests carried out for the secondary fixing following on from the primary fixing with paraformaldehyde, it is theoretically possible to use other reagents which render the cell membrane slightly permeable, such as detergents, for example.

The transient expression of genes which modulate apoptosis, for example members of the Bcl family or components of the survival factor signal transduction, and the subsequent quantitative analysis of apoptosis using the method according to the invention make it possible to test chemical compounds to see whether they are capable of specifically influencing the function of apoptosis-modulating genes.

The method according to the invention can be automated by a suitable adaptation of apparatus, e.g. the preparation of samples and the FACS analysis, which makes it suitable for carrying out measurements on a large scale, e.g. in High Throughput Screening methods.

The method in this form is used in the identification of pharmaceutically active substances which are able to modulate apoptosis as a function of the expression of certain genes (apoptosis test genes). The gene whose effect on apoptosis is supposed to be modulated by the test substance is transiently transfected into test cells and the test cells are incubated with a range of substances. The modulating effect of a test substance

on the activity of the test gene is measured directly using measuring instruments.

Methods of this kind can be used for the following screening applications:
a) searching for inhibitors of survival factors and their signal transduction, as well as inhibitors of anti-apoptotic gene products in tumour cells; b) searching for chemicals which, synergistically with chemotherapy, inhibit certain survival factors and their signal transduction in tumour cells; c) searching for chemotherapeutic agents which act synergistically with the inhibition of survival factors.

Moreover, the method according to the invention can be used to investigate known genes as to whether and to what extent they modulate apoptosis in different types of cells.

Another use for the method according to the invention is the expression cloning of genes which modulate apoptosis. For this, a complete cDNA expression library is transiently transfected into cells. The method according to the invention is capable of measuring the influence of gene expression within 24 to 48 hours. It is therefore possible to analyse and isolate cells while they are still alive. For this purpose the method is modified by using FACS sorting to isolate single cells which deviate from an apoptosis background which is to be determined. The plasmids transfected into these cells are isolated, amplified and selected in further transfection processes. Plasmids which contain an apoptosis-modulating gene are thus isolated. The corresponding genes are then characterised by sequencing and other studies of expression and function.

To validate the method according to the invention, first of all, in Example 1, established tumour cell lines were used which had been transfected on the one hand with a GFP plasmid and on the other hand with a plasmid containing a pro-apoptotic or an anti-apoptotic gene sequence, or a control plasmid. After the transfection the cells were treated, after a period of rest, with an apoptotic stimulus (control cells remained untreated). Then the detached cells were collected and combined with the trypsinised adherent cells, washed and fixed. After being washed the cells were divided up to make it possible to compare the method according to the invention with the conventional TUNEL method which uses fluorescent Cy5-dCTP (5-amino-propargyl-2'-deoxycytidine 5'-triphosphate coupled to Cy5 fluorescent stain).

It has been found that the method according to the invention reliably detects the expected pro- or anti-apoptotic effect of the gene products and that the addition of the apoptotic stimulus intensifies the effect observed. Although the sensitivity of the method according to the invention under the conditions selected was slightly less than that of the TUNEL method, the standardised apoptosis value, expressed as the ratio of the maximum apoptosis values of the particular assay achieved with the pro-apoptotic gene, was virtually identical. Compared with the TUNEL method the method according to the invention has the advantage of speed, simplicity and cheapness.

The versatility and reliability of the method according to the invention were confirmed by the use of an untransformed rat fibroblast cell line which reacts

less to apoptotic stimuli than the established tumour cell lines.

The method according to the invention makes it possible to establish the potential role of a gene product in apoptosis rapidly, effectively and reproducibly.

According to another aspect, the invention relates to a kit for carrying out the process simply as a routine procedure.

A kit of this kind will expediently contain the following components in a number of separate containers:

a) one or more components required for the transfection;
b) a plasmid containing the sequence coding for the fluorescent marker protein;
c) an empty vector for inserting the DNA sequence of particular interest and for control measurements;
d) the primary fixing solution, e.g. paraformaldehyde solution;
e) the secondary fixing/permeabilising solution, e.g. 70 % ethanol;
f) washing solution(s);
g) a DNA-binding stain.

Preferably, the kit contains polyethyleneimine and psoralen/UV-inactivated Adenovirus as transfection components.

Example 1

For this Example, established tumour cell lines (βTC and βHC) were used, derived from β-cell tumours (15) in transgenic mice, in which the regulatory region of the insulin gene (Rip) specifically induces the expression of the large T-antigen of Simian virus 40 (Tag) in the β-cells of pancreatic islets (16).

About 80,000 cells were seeded into a 6 cm well in a 6-well culture dish and cultivated in DMEM, supplemented with 10 % FCS (v/v), 2 mM glutamine, 100 International Units of penicillin and 100 µg/ml of streptomycin, until 70 % confluence was achieved. The cells were transfected with 1 µg of a plasmid coding for eGFP ("enhanced GFP"; pEGFP-C1; Clontech) together with 1 µg of a control plasmid (pMEX; (22)), a pCMV plasmid containing the pro-apoptotic Adenovirus gene E1A or a pCMV plasmid containing the anti-apoptotic Adenovirus gene E1B-19K (17, 18) using 10 µl of LipofectAMINE (GIBCO-BRL) in accordance with the manufacturer's recommendations. After the transfection the cells were left to stand for 16 h in complete medium, then the cells were either left untreated or treated for a further 16 h with an apoptotic stimulus (800 ng/ml of staurosporin; Sigma) (19, 20). 32 h after the transfection the detached cells were combined with trypsinised, adherent cells, washed twice with 4 ml of PBS and fixed at ambient temperature for 30 min (2 % paraformaldehyde, 100 mM NaCl, 300 mM saccharose, 3 mM $MgCl_2$, 1 mM EGTA (ethyleneglycol-bis-(2-aminoethyl)-tetraacetic acid), 10 mM PIPES (piperazine-N_1N^1-bis[z-ethanesulphonic acid]) pH 6.8). Then they were washed twice with 4 ml of PBS and fixed for 14 h in ice-cold 70 % EtOH.

After the fixing, the cells were washed twice with 4 ml of PBS and divided up. One half of the sample was treated with RNase A (Sigma, St. Louis, USA)(50 µg/ml) in PBS for 30 min, washed twice with 4 ml of PBS and, 30 min before the FACS analysis, stained with propidium iodide in PBS (PI; 50 µg/ml; Sigma, St. Louis, USA). The other half of the sample was incubated for 1 hour at 37°C with 50 µl of TdT reaction mixture (terminal deoxynucleotidyl transferase; Boehringer Mannheim; 200 mM potassium cacodylate, 25 mM of Tris-HCl, pH 6.6, 0.25 mg/ml bovine serum albumen, 1 mM $CoCl_2$; 0.25 nmol FluoroLink Cy5AP3-dCTP [Amersham], 12.5 units of TdT), washed twice with 4 ml of PBS, treated with RNase in PBS (50 µg/ml) for 30 min, washed twice with 4 ml HBS (from this step onwards HBS was used instead of PBS because DAPI has a tendency to produce microprecipitates in PBS), stained with DAPI in HBS (10 µg/ml; Sigma) for 20 min and analysed using a Becton Dickinson FACS Vantage apparatus. The FACS analysis of the PI-stained cells was carried out with a Becton Dickinson FACScan apparatus fitted with a so-called "doublet discrimination module", with which cell aggregates are discriminated by calculating the pulse width and pulse width. The results of the tests are shown in Fig. 1. Fig. 1A shows the number of apoptotic βHC 13T tumour cells (% apoptosis) in the entire eGFP-positive cell population. The black bars indicate the determination of the sub-2N DNA content (GFP/PI); the white bars indicate the incorporation of fluorescent Cy5AP3-dCTP during the TdT reaction (GFP/TUNEL). The addition of staurosporin is shown. An excitation wavelength of 488 nm was used for eGFP and PI, an excitation wavelength of 647 nm was used for Cy5 and UV of a wavelength range of 51 - 364 nm was used for DAPI. The emission fluorescence was collected using a 530/20 nm narrow band filter for eGFP, a 610 nm

blocking filter for PI, a 675/20 nm narrow band filter for Cy5 and a 424/44 narrow band filter for DAPI. Doublets were excluded by means of pulsed processing. eGFP-expressing cells were selected and analysed for Cy5- or PI-fluorescence. The data were analysed using CELLQuest software (Becton Dickinson). Each bar represents the average of 3 transfections, standard deviations are indicated by error bars. Each measurement comprised 40,000 total events, selected according to size and single cells. The transfection efficiency was 20-30 %.

Fig. 1B shows the standardised percentage of apoptosis for the various constructs. The apoptosis index was standardised using the following function: (% apoptosis in X/% apoptosis in eGFP-C1/E1A) x 100. The apoptotic index was standardised for each of the detection methods used and for each subsequent transfection treatment (+/- staurosporin).

Example 2

In this Example an untransformed rat fibroblast cell line designated Rat1A was used. The cells were transiently transfected using either LipofectAMINE, as described in Example 1, or polyethyleneimine (PEI 2000)-DNA-Adenovirus complexes (WO 93/07283). Moreover, regarding the treatment of the cells and determination of apoptosis, using the process according to the invention on the one hand and the TUNEL method on the other hand, the procedure was exactly as described in Example 1. A comparison of the different transfection methods and methods of measuring apoptosis is shown in the Table. Each value represents the average of 3 transfections; the standard deviation is given (s.d.). The efficiency of both transfection methods was 25-30 %.

Table

Trans-fected Construct	**LipofectAMINE**			
	Propidium iodide		**TUNEL**	
	% apoptosis (s.d.)	% apoptosis standardised	% apoptosis (s.d.)	% apoptosis standardised
pMEX (Ctr)	3.3 (0.7)	68.8	6.5 (0.6)	71.4
pE1B-19K	1.4 (0.3)	29.2	2.2 (0.5)	24.2
pE1A	4.8 (0.4)	100	9.1 (2.6)	100
	PEI / Adeno			
pMEX (Ctr)	1.2 (0.2)	52.2	7.5 (0.3)	52.1
pE1B-19K	0.7 (0.1)	30.4	5.2 (0.8)	36.1
pE1A	2.3 (0.6)	100	14.4 (2.7)	100

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Patent Claims

1) Process for measuring apoptosis, characterised in that

A) a population of mammalian cells is transiently transfected

ai) with a plasmid containing a DNA sequence of interest which is to be investigated as to whether it or the polypeptide expressed thereby has a pro- or anti-apoptotic activity,

aii) or with a plasmid containing a DNA sequence of interest which is to be investigated as to whether, or by means of which substances, its pro- or anti-apoptotic activity or the activity of the polypeptide expressed thereby can be modulated,

b) and with a plasmid containing a DNA coding for a fluorescent marker protein,

B) in that the cells are incubated in a suitable nutrient medium, until the DNA sequence of interest or the expressed polypeptide has exerted its potential activity on the apoptosis,

C) in that the cells are harvested and fixed so that the fluorescent protein remains in the cells, while the DNA fragments formed during apoptosis are able to diffuse out of the cells,

D) in that the proportion of apoptotic cells is determined by measuring the DNA content,

E) in that the proportion of transfected cells is determined by measuring the cells with fluorescent marker protein,

F) and that by comparing the values obtained in steps D and E the proportion of apoptotic cells in the transfected subpopulation of the cells is determined.

2. Process according to claim 1, characterised in that the transfection of the cells is carried out with polyethyleneimine and inactivated Adenovirus.

3. Process according to claim 1, characterised in that the fluorescent polypeptide defined in A b) is Green Fluorescent Protein.

4. Process according to claim 1, characterised in that the DNA content is measured with a DNA-binding stain.

5. Process according to claim 4, characterised in that the stain is propidium iodide.

6. Process according to one of claims 1 to 5, characterised in that the incubation according to step B) is carried out in the presence of a test substance.

7. Process according to one of claims 1 to 6, characterised in that the incubation according to step B) is carried out in the presence of a substance which stimulates apoptosis.

8. Process according to one of claims 1 to 7, characterised in that the primary fixing in step C is carried out with paraformaldehyde and the secondary fixing/permeabilisation of the cells is carried out with ethanol.

9. Process according to one of claims 1 to 8, characterised in that the measurements defined in steps D and E are carried out in one step using fluorescence-activated throughflow cytometry analysis.

10. Kit for performing the process according to claim 1, characterised in that it contains the following components in several separate containers:

a) one or more components required for the transfection;

b) a plasmid containing the sequence coding for the fluorescent marker protein;

c) an empty vector for inserting the DNA sequence of particular interest and for control measurements;

d) the primary fixing solution;

e) the secondary fixing/permeabilising solution;

f) washing solution(s);

g) a DNA-binding stain.

11. Kit according to claim 10, containing as component a) polyethyleneimine and psoralen/UV-inactivated Adenovirus.

12. Kit according to claim 10, containing as component b) a plasmid coding for Green Fluorescent Protein.

13. Kit according to claim 10, containing as component d) an approximately 2% paraformaldehyde solution and as component e) about 70 % ethanol.

14. Use of the process according to claim 1 for identifying substances which modulate the pro- or anti-apoptotic activity of genes or gene products.

Abstract

Process for measuring apoptosis quickly and easily. Mammalian cells are co-transfected with a plasmid which codes for a fluorescent protein, and with a plasmid carrying a gene of interest. After incubation and gentle fixing, apoptosis is measured by determining the DNA content of the cells using DNA-binding stain and determining the proportion of transfected cells by throughflow cytometry.